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Elevated Levels of Tumor Necrosis Factor-Beta, Gamma-Interferon, and IL-6 mRNA in Castleman's Disease

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Castleman's disease (CD) is a lymphoproliferative disorder characterized by enlarged hyperplastic lymph nodes. CD may be localized or multifocal, and is often associated with signs and symptoms of generalized inflammation. The systemic manifestations of CD have been previously attributed to an overproduction of interleukin-6 (IL-6) by the tumor, although there is evidence that IL-6 is not responsible for all of the symptoms. We describe a 9-year-old boy who developed Castleman's disease with systemic findings of hypochromic microcytic anemia, growth arrest, inflammation, and hyperimmunoglobulinemia. Following surgical resection, all of the symptoms and laboratory abnormalities resolved. Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the tumor, we found elevated levels of IL-6 mRNA as expected, but also elevated levels of tumor necrosis factor beta (TNF-β) and gamma interferon (γ-IFN) mRNA. Because these cytokines are mediators of immune regulation and inflammation, we propose that TNF-β and γ-IFN also play an important role in the pathophysiology of Castleman's disease.

Key words: Castleman's disease, cytokines, interleukin-6, gamma-interferon, tumor necrosis factor-beta

INTRODUCTION

Castleman's Disease (CD), also known as angiofollicular hyperplasia or lymphoid hamartoma, is a lymphoproliferative disorder that can affect both children and adults. Castleman et al. first described a group of patients with large, benign mediastinal hyperplastic lymph nodes [1] and later divided CD into two categories, hyaline vascular and plasma cell disease, based on histological criteria [2]. Either localized or multifocal, CD is typically associated with a number of signs and symptoms, including hypochromic microcytic anemia, growth arrest, polyclonal hypergammaglobulinemia, and systemic signs of inflammation, including fever, elevated erythrocyte sedimentation rate (ESR) and C-reactive protein [1–5]. In patients with localized CD, complete resection of the tumor mass can result in prompt amelioration of all systemic symptoms and all laboratory abnormalities [5,6].

Elevated serum levels of interleukin-6 (IL-6) have been previously described in CD [7], and correlate with the severity of constitutional symptoms [8]. By in situ hybridization, IL-6 originates from the B cells and reticular dendritic cells in the affected lymph node [7,9]. While IL-6 can elicit many symptoms of the acute phase response [10], it may not be responsible for all of the clinical and laboratory abnormalities observed in CD. One patient with CD and highly elevated serum levels of IL-6 was recently treated with a monoclonal anti-IL-6 antibody; his fever and constitutional symptoms improved within 24 hours, and many of his abnormal laboratory values improved within two weeks. However, his hypochromic anemia, thrombocytosis and elevated C-reactive protein only partially improved, and a sustained remission was achieved only after surgical resection [5].

Experiments with transgenic mice have demonstrated that IL-6 over-expression leads to a typical pathological pattern, including an infiltrating polyclonal plasmacytosis, mesangioproliferative glomerulonephritis, and an increased number of bone marrow megakaryocytes [11,12]. While minor differences were noted depending upon the tissue over-expressing IL-6, no mouse strain developed a hypochromic microcytic anemic or symptoms of inflammation. In addition, mice with hematopoietic progenitors infected with an IL-6-expressing N2 ret-

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TABLE I. Laboratory Parameters for the Patient With Castleman's Disease*

	Pre-operative dates		Post-operative dates	
Parameter	Jan. 25, 1994	Feb. 14, 1994	May 10, 1994	Dec. 2, 1994
Hemoglobin (gm/dL)	6.7	7.1	10.1	12.9
Hematocrit (%)	22	24	33	38
Reticulocytes (%)	1.1		0.3	
Platelets ($\times 10^9$/L)	672	611	456	224
MCV (fL)	48	50	60	74
Serum iron (μg/dL)	2	17	35	
TIBC (μg/dL)	272	285	390	
Ferritin (ng/ml)	6	11	17	
ESR (mm/hour)	94	80	14	5
Albumin (gm/dL)		3.4	4.0	
Total protein (gm/dL)		8.0	7.8	

*Surgical excision occurred on April 22, 1994; the patient received one unit packed erythrocytes intraoperatively. MCV = mean corpuscular volume, TIBC = total iron binding capacity, ESR = erythrocyte sedimentation rate.

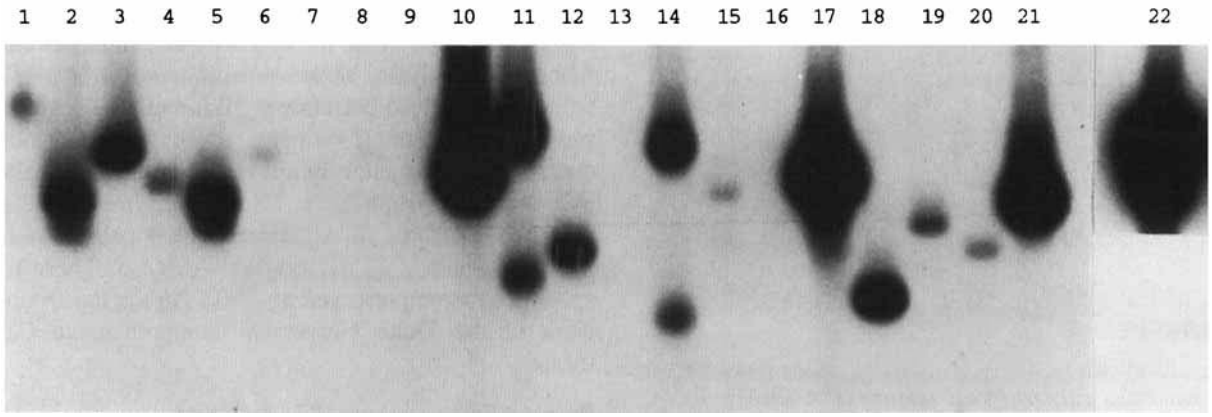

Fig. 1. Analysis of cytokine mRNA expression from the Castleman's tumor. Total RNA was extracted from the tumor mass and analyzed by RT-PCR as described in Materials and Methods. Each cytokine mRNA signal is shown in adjacent lanes on the autoradiogram. Lane 1 = TNF-α; Lane 2 = TNF-β; Lane 3 = γ-IFN; Lane 4 = IL-1α; Lane 5 = IL-1β; Lane 6 = IL-2; Lane 7 = IL-3; Lane 8 = IL-4; Lane 9 = IL-5; Lane 10 = IL-6; Lane 11 = IL-7; Lane 12 = IL-8; Lane 13 = IL-9; Lane 14 = IL-10; Lane 15 = IL-11; Lane 16 = IL-13; Lane 17 = TGFβ1; Lane 18 = TGF β2; Lane 19 = G-CSF; Lane 20 = GM-CSF; Lane 21 = M-CSF; and Lane 22 = β-actin control.

rovirus had some of the clinical manifestations of CD, but did not have adenopathy or effacement of nodal architecture [13]. None of these experimental mouse models completely portrayed the features of CD, suggesting that cytokines other than IL-6 may be involved in the pathophysiology of this disease.

In this report, we describe a child who presented with anemia, growth arrest, and hypergammaglobulinemia who was found to have localized CD. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the tumor mass revealed highly elevated levels of IL-6 mRNA as expected, but also elevated levels of tumor necrosis factor beta (TNF-β) and gamma interferon (γ-IFN) mRNA. Similar RT-PCR analysis of a nonlymphoid hamartoma and a normal lymph node demonstrated only minimal amounts of IL-6, TNF-β, and γ-IFN mRNA, suggesting that these three cytokines might be important in the pathophysiology of CD. However, other lymphoid neoplasms had varying mRNA levels of IL-6, TNF-β, and γ-IFN, as well as differing patterns of clinical presentation. We conclude that in addition to IL-6, TNF-β and γ-IFN contribute to the pathophysiology and constitutional symptoms observed in CD, and that these cytokines may be important in other lymphoproliferative disorders as well.

CASE REPORT

A 9-year-old white male presented for evaluation of profound microcytic hypochromic anemia unresponsive to a 3-month trial of iron therapy, and a 6-month period of growth arrest. Past medical history was significant only for aberrant pulmonary venous return from the right upper lobe (Scimitar syndrome) diagnosed in infancy, when a complete blood count was normal. At presentation, the patient's physical exam was significant for pal-

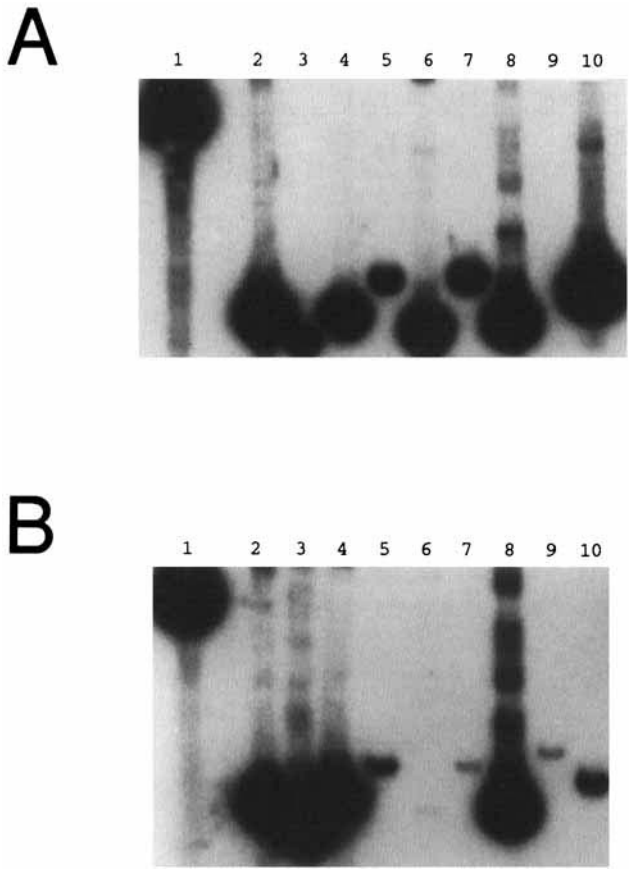

Fig. 2. Comparison of cytokine mRNA expression between Castleman's tumor and a non-lymphoid hamartoma. A selective RT-PCR cytokine profile was performed on total RNA extracted from the tumor mass **(A)** and the hamartoma **(B)**. Lane 1 = β-Actin; Lane 2 = TGF-β1; Lane 3 = TGF-β2; Lane 4 = IL-1β; Lane 5 = TNF-α; Lane 6 = TNF-β; Lane 7 = γ-IFN; Lane 8 = M-CSF; Lane 9 = IL-9; and Lane 10 = IL-6. The results show that the elevated levels of TNF-β, γ-IFN, and IL-6 mRNA are specific for the Castleman's tumor.

lor without palpable hepatosplenomegaly or enlarged lymph nodes. His weight and height were in the 5th percentile and 10th percentiles for his age, respectively. The initial laboratory values are listed in Table I. The patient had a severe microcytic anemia with evidence of iron deficiency, and an elevated ESR. His serum erythropoietin level was elevated at 105 IU/L (normal <24). Serum protein electrophoresis revealed a polyclonal hyperimmunoglobulinemia: serum IgG = 2,550 mg/dl (normal 639–1,349), IgA = 377 mg/dl (normal 11–90), and IgM = 252 mg/dl (normal 34–126). Examination of the bone marrow revealed a myeloid to erythroid ratio of 1.5:1.0, increased numbers of plasma cells, and signs of ineffective erythropoiesis. Stainable iron was predominantly localized to macrophages, and ringed sideroblasts were not identified.

A chest X-ray did not show adenopathy, but an abdominal CT revealed a mass in the region of the tail of the pancreas. At laparotomy, a 6 × 5 × 2 cm retroperitoneal encapsulated mass weighing 51 g was fully resected. Pathological examination showed giant lymph node hyperplasia of mixed plasma cell and hyaline vascular histology (Castleman's disease). Postoperative recovery was uneventful. The patient experienced rapid improvement in all of his hematological abnormalities, normalization of the ESR to 5 mm/hr, and a "catch up" growth spurt of 1.3 kg over 3 months (Table I). He is currently asymptomatic for any of his presenting clinical and laboratory abnormalities 10 months after excision of the tumor mass.

MATERIALS AND METHODS

Tissue Samples

Samples of the patient's tumor were collected intraoperatively using sterile surgical technique and immediately frozen at −80°C. Due to the retrospective nature of this laboratory analysis, serum samples were not available from the patient to correlate cytokine mRNA levels with protein expression. Two tissue samples used as normal controls included a non-lymphoid intracranial hamartoma and a normal lymph node. Neoplastic lymph node samples were obtained from patients with T-cell lymphoma, B-cell lymphoma, and Hodgkin's disease. These tissue specimens were preserved at −80°C in the tissue storage bank of the Duke University Comprehensive Cancer Center.

Reverse Transcriptase (RT) Reaction

Each tissue sample was thawed at room temperature and lysed in 4 M guanidinium buffer, and total RNA was isolated using a cesium chloride centrifugation method [14]. Total RNA (1 μg) was then converted into cDNA template using random hexamer primers, as previously described [15].

Primers

Oligonucleotide primers were synthesized according to the published cDNA sequences for a panel of cytokines. Primer pairs were designed to amplify the following cytokines and growth factors: TNF-α, TNF-β, γ-IFN, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, transforming growth factor beta 1 (TGF β1), TGF β2, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), and monocyte stimulating factor (M-CSF). β-Actin served as a positive control.

Polymerase Chain Reaction (PCR)

PCR reactions were run in parallel for each primer pair using reagents and conditions as previously described [15]. Briefly, each reaction contained 100 ng of RT

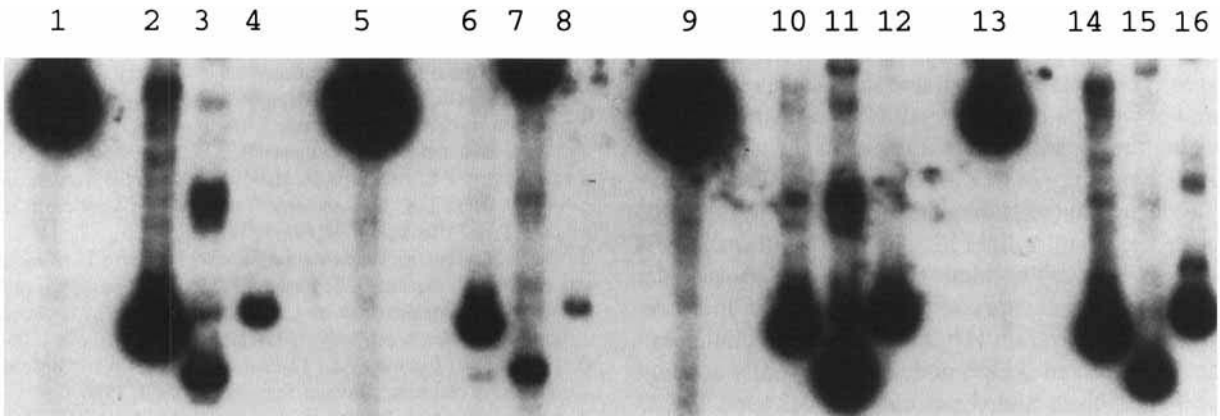

Fig. 3. Comparison of cytokine mRNA expression in CD and other lymphoid malignancies. RT-PCR mRNA levels of β-actin, IL-6, TNF-β, and γ-IFN, respective from left to right, are shown for each tumor tissue in the panel. Analysis was performed on total RNA from the Castleman's disease (Lanes 1–4), B-cell lymphoma (Lanes 5–8), angioimmunoblastic T-cell lymphoma (Lanes 9–12), and Hodgkin's disease (Lanes 13–16).

cDNA, 1 μg of both primers, 100 pm of each nucleotide, and 12.5 μCi of [α-^{32}P]dCTP, and was cycled at 94°C for 30 seconds, 60°C for 30 seconds, then 72°C for 60 seconds on a Perkin Elmer (Norwalk, CT) DNA Thermal Cycler. Samples were removed after 30 cycles. Five μl aliquots of PCR products were electrophoresed through a 6% polyacrylamide gel, dried for 2 hours at 50°C (Hoefer Scientific Instruments, San Francisco, CA), and exposed to X-omat film (Eastman Kodak Co, Rochester NY) with an intensifying screen at −80°C.

RESULTS

The complete RT-PCR screen on the patient's CD tumor tissue revealed a complex cytokine mRNA profile shown in Figure 1. As expected, the Castleman's tumor expressed high levels of IL-6 mRNA (Lane 10), but also significant levels of TNF-β (Lane 2), γ-IFN (Lane 3), IL-1β (Lane 5), TGF-β1 (Lane 17), TGF-β2 (Lane 18), and M-CSF mRNA (Lane 21).

To test whether or not production of these cytokine mRNA species was specific for Castleman's tumor, we also performed RT-PCR cytokine analysis on a non-lymphoid hamartoma (Fig. 2). Using a more restricted set of cytokine primers, the non-lymphoid hamartoma had substantial mRNA expression for some of these cytokines (TGF-β1, TGF-β2, IL-1β, and M-CSF). However, the hamartoma had only minimal expression of TNF-β, γ-IFN, and IL-6 mRNA, unlike the Castleman's tumor. Similarly, normal lymph node tissue expressed very low amounts of TNF-β, γ-IFN, and IL-6 mRNA (data not shown).

RT-PCR analysis was also performed on three neoplastic lymph node specimens (Fig. 3). Expression of IL-6, TNF-β, and γ-IFN mRNA was elevated in the CD tumor (Lanes 2–4), but relatively lower in a well-differentiated B-cell lymphoma (Lanes 6–8). Elevated levels for all three cytokines were identified in an angioimmunoblastic T-cell lymphoma (Lanes 10–12) and stage IVB Hodgkin's disease (Lanes 14–16).

DISCUSSION

RT-PCR cytokine analysis of our patient's tumor revealed elevated levels of IL-6 mRNA when compared to a normal lymph node and a non-lymphoid hamartoma. These control tissue samples were used to identify cytokines produced by normal lymphoid cells and stroma. In addition to IL-6, however, the CD tumor expressed elevated levels of γ-IFN and TNF-β mRNA. These two cytokines play important roles in immune regulation, and have biological actions that may help explain the clinical findings of fever, cachexia, autoimmune disease, and abnormal lymph node architecture found in CD and other lymphoproliferative disorders.

Tumor necrosis factor beta (lymphotoxin) is released by T lymphocytes and mediates inflammation. It has similar biological activity to TNF-α; in fact, these two cytokines can activate the same receptor [16,17]. TNF-β is also critical for lymphoid organogenesis; mice deficient in TNF-β did not develop peripheral lymph nodes and had abnormal splenic architecture [18]. Overproduction of TNF-β may therefore contribute to the adenopathy and abnormal nodal architecture seen in CD and other lymphoproliferative disorders. TNF-β also has been shown to work synergistically with γ-IFN to inhibit erythroid colony formation [19,20], induce monocytoid differentiation [21], and induce IL-6 production in human fibroblasts [22].

Gamma interferon is produced by activated lymphocytes, and is a potent activator of monocytes and macrophages. The fever, myalgia, and fatigue encountered in CD are known side effects of γ-IFN administration [23], suggesting the biological significance of this cytokine in the pathophysiology of CD. Both γ-IFN and TNF-β have been implicated in the pathogenesis of cytokine-mediated cancer cachexia and weight loss [17]. In addition, γ-IFN and TNF-β both up-regulate the synthesis of vascular adhesion molecules, especially ELAM-1, which are required for cell migration [16,23]. Increased cellular migration into affected lymph nodes may partially account for the hyperplastic and dysmorphic nodes seen in CD [9]. Importantly, these histologic findings were not seen in the IL-6 retroviral mouse model [13]. Finally, γ-IFN has also been hypothesized to play a role in the pathogenesis of autoimmune disease [16,23,24]. Our patient did not manifest clinically overt autoimmune symptoms, but several authors have noted the occurrence of autoimmune disease in CD [3,4].

Given the powerful biological effects of TNF-β and γ-IFN, dysregulation in the synthesis of these cytokines may play an important role in the pathophysiology of many lymphoproliferative diseases [17,25–27]. Our data demonstrate highly elevated TNF-β and γ-IFN mRNA levels in CD but also in angioimmunoblastic T-cell lymphoma and Hodgkin's disease (Fig. 3). These two latter patients experienced fatigue, fever, night sweats and loss of appetite at the time of lymph node biopsy. In contrast, the patient with well-differentiated B-cell lymphoma did not display these clinical features, and had relatively low levels of cytokine production (Fig. 3), consistent with previous results [27].

It is not clear whether these cytokines are produced directly by the neoplastic cells or secondarily by reactive lymphocytes within the diseased lymph node. Investigation using in situ hybridization may shed light on this important question [7,17]. It is also difficult to infer a direct cause-and-effect relationship between cytokine mRNA dysregulation and the systemic features found in CD and other lymphoproliferative disorders. Nevertheless, our data suggest that dysregulation and hypersecretion of multiple cytokines play an important role in the pathophysiology of CD, as has already been reported for other lymphoid tumors [27].

In summary, we present direct experimental evidence that, in addition to IL-6, TNF-β and γ-IFN mRNA are expressed at high levels in CD tumor tissue. The combined effects of γ-IFN and TNF-β likely cause some of the systemic clinical manifestations of CD that are not attributable to IL-6 alone.

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